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DGKζ ablation engenders upregulation of p53 level in the spleen upon whole-body ionizing radiation

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ABSTRACT

The tumor suppressor gene product p53, which coordinates the cellular response to various stresses, is subject to tight regulation by a complex network of signal transduction. The DGK family metabolizes lipidic second messenger diacylglycerol to produce phosphatidic acid. Our earlier studies showed that one isozyme, DGKζ, is involved in the regulatory mechanism of p53. In a cellular model of doxorubicin-induced DNA damage, overexpression of wild-type DGKζ suppresses p53 protein induction and reduces apoptosis, whereas knockdown of DGKζ upregulates p53 protein level and promotes apoptosis. Further examination reveals that DGKζ facilitates p53 degradation via ubiquitin-proteasome system in the cytoplasm. However, it remains undetermined whether the regulatory mechanism of DGKζ on p53 function found in cell-based experiments is also functional at the animal level. This study was conducted to elucidate this point using an experiment with DGKζ-KO mice under DNA damage induced by whole-body ionizing radiation. Our results reveal that p53 protein is induced robustly in the spleen of DGKζ-KO mice upon exposure to ionizing radiation, thereby promoting apoptosis in this organ. Taken together, the results demonstrate that DGKζ plays a sentinel role in p53 expression at the cellular and organismal levels after DNA damaging stress conditions.

1. Introduction

Diacylglycerol kinase (DGK) is an enzyme that phosphorylates diacylglycerol (DG) to produce phosphatidic acid (PA) in lipid metabolism (Kanoh et al., 1990). In this process, DGK serves as a pivotal regulator of signal transduction by DG signal attenuation and PA signal initiation, thereby regulating the balance between the two actions. Therefore, DGK is deeply involved in lipidic signal transduction cascade together with DG-generating phospholipase C (PLC) and PA-metabolizing enzymes such as PA phosphatase (Brindley et al., 2002; Sciorra and Morris, 2002).

Reportedly, DGK consists of several isozymes, each of which is involved in characteristic functions and each of which acts at a subcellular site such as the plasma membrane, endoplasmic reticulum, cytoskeleton, and nucleus (Goto et al., 2007; Sakane et al., 2007; Topham and Epand, 2009). The physiological roles of mammalian DGK family have been described in the fields of immune system, cancer biology, and neuronal function (Mérida et al., 2017; Riese et al., 2016; Tu-Sekine et al., 2015). One isozyme, DGKζ, is unique in that it contains both a nuclear localization signal (NLS) (Goto and Kondo, 1996) and a nuclear export signal (NES)

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(Evangelisti et al., 2010). An earlier study demonstrated that DGKζ translocates from the nucleus to the cytoplasm and that it is degraded in hippocampal neurons under stress conditions including ischemia (Ali et al., 2004), kainate-induced seizures (Saino-Saito et al., 2011), and oxygen glucose deprivation (OGD) (Suzuki et al., 2012). These findings indicate that cytoplasmic translocation and degradation of DGKζ are closely involved in the pathogenesis of neuronal stress such as glutamate excitotoxicity (Okada et al., 2012).

Additional studies have revealed that DGKζ plays an important role in the regulation of p53. The protein p53, a master molecule in cellular stress responses, is multifunctional. It is activated in response to genotoxic stress to stimulate the expression of genes that cause cell cycle arrest or apoptosis, depending on the damage severity (Polager and Ginsberg, 2009; Vousden and Prives, 2009). In addition to this role as a nuclear transcriptional factor, cytoplasmic p53 associates with the proapoptotic protein Bak at the mitochondria to trigger apoptosis in a transcription-independent manner (Green and Kroemer, 2009; Marchenko et al., 2007).

In this regard, DGKζ promotes nuclear p53 transcriptional activity and its degradation in the cytoplasm via ubiquitin-proteasome system (UPS) (Goto et al., 2014; Tanaka et al., 2013). Therefore, cytoplasmic translocation of DGKζ suppresses p53 cytotoxicity through both attenuation of the transactivation activity in the nucleus and the protein degradation in the cytoplasm. However, DGKζ itself also undergoes degradative breakdown by UPS; downregulation of DGKζ engenders accumulation of p53, which induces cytotoxicity in the cytoplasm (Okada et al., 2012).

Consequently, overexpression of wild-type DGKζ is shown to suppress p53 induction and to reduce apoptosis, whereas its knockdown promotes apoptosis in HeLa cells after doxorubicin-induced DNA damage (Tanaka et al., 2013). In animal experiments, neurons that are deficient of DGKζ do not show apoptosis directly, although they are more vulnerable to excitotoxicity than wild-type neurons are (Okada et al., 2012). Furthermore, cardiac-specific overexpression of DGKζ affects beneficial effects on a pressure-overloaded or infarcted heart and protects apoptosis in cardiomyocytes, thereby increasing the survival rate compared with that of wild-type mice (Niizeki et al., 2007, 2008). These findings suggest that DGKζ is intimately involved, in a protective manner, in the regulatory mechanism of apoptosis.

X-ray irradiation-induced DNA double-strand break is a major event regulated by p53; it is an initiator of radiation-induced genomic instability (Chang and Little, 1992; Suzuki et al., 2003). DNA double-strand break is recognized by ataxia telangiectasia mutated kinase (ATM) (Nelson and Kastan, 1994), which promotes the accumulation and activation of p53, CHK2/Cds1, BRCA1, NBS1, and histone H2AX. Especially, p53, a well-known tumor suppressor protein, induces transactivation of diverse downstream genes such as p21, GADD45, Bax, and p53AIP1, thereby leading to cell cycle arrest, apoptosis, and DNA repair in a context-dependent manner (Vousden and Prives, 2009). Nevertheless, it remains unclear whether the regulatory mechanism of DGKζ on p53 function observed in cell-based experiments is also functional at the animal level. To address this issue, we conducted an experiment of DGKζ-deficient mice under whole-body irradiation, the standard induction of p53 protein, and examined the spleen, an organ that is extremely sensitive to X-ray irradiation. We show here that p53 protein is induced strongly in DGKζ-deficient spleen when exposed to whole-body ionizing radiation, confirming that DGKζ exerts a negative regulation on p53 expression at the animal level.

2. Materials and methods

2.1. Animals

All experiments using rats and mice were carried out in accordance with the guidelines and the permission of the Animal Research Center of Yamagata University. C57BL6 mice (wild-type) were purchased from Japan SLC Inc. DGKζ knockout (KO) mice used in the present study are global knockouts (Zhong et al., 2003). Wild-type and DGKζ-KO mice were bred and maintained with sterilized food, water and bedding at the animal facility of the Yamagata university school of medicine.

2.2. Immunoblotting

Under anesthesia with ether, organs and tissues were rapidly removed and homogenized in a lysis buffer containing 20 mM Tris–acetate (pH 7.4), 100 mM potassium acetate, 0.5% Triton X-100, 1 mM Na_3VO_4, 1 mM NaF, protease inhibitors cocktail (Sigma), and centrifuged at 16,000 *g* for 10 min at 4 ˚C to remove debris. The protein concentration of the resulting supernatant was determined using the BCA protein assay kit (Pierce). The resulting supernatant was boiled for 10 min in SDS sample buffer (New England Biolabs) and subjected to 10% SDS–PAGE. The proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (NEN™ Life Science Products). After blocking the non-specific binding sites with 4% non-fat dry milk in phosphate-buffered saline (PBS) containing 0.2% Tween 20, the membrane was incubated for overnight at 4 ˚C with guinea pig anti-DGKζ antibody (Hozumi et al., 2003), p53 (1:1000, sc-6243G, Santa Cruz), PARP (1:1000, 9542, Cell Signaling), Mdm2 (1:1000; clone Ab-1; Calbiochem), β-actin (1:5000, A5441, Sigma) in PBS containing 0.1% Tween 20. After washing with PBS-T, the membranes were incubated with peroxidase-linked secondary antibodies for 1 h at room temperature (1:5000, GE Healthcare). After 3 washes, immunoreactive bands were visualized with a chemiluminescence detection kit (Millipore).

2.3. Immunohistochemistry

For immunohistochemistry, animals were anesthetized with ether and fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for immunofluorescence microscopy. Frozen spleen sections of 20 μm thickness were cut using a LEICA CM1900 cryostat (Leica Microsystems). All immunohistochemical incubations were performed at room temperature. Spleen sections were incubated in 10% normal donkey serum for blocking and incubated with p53 (1:100, sc-6243G, Santa Cruz) or cleaved caspase3